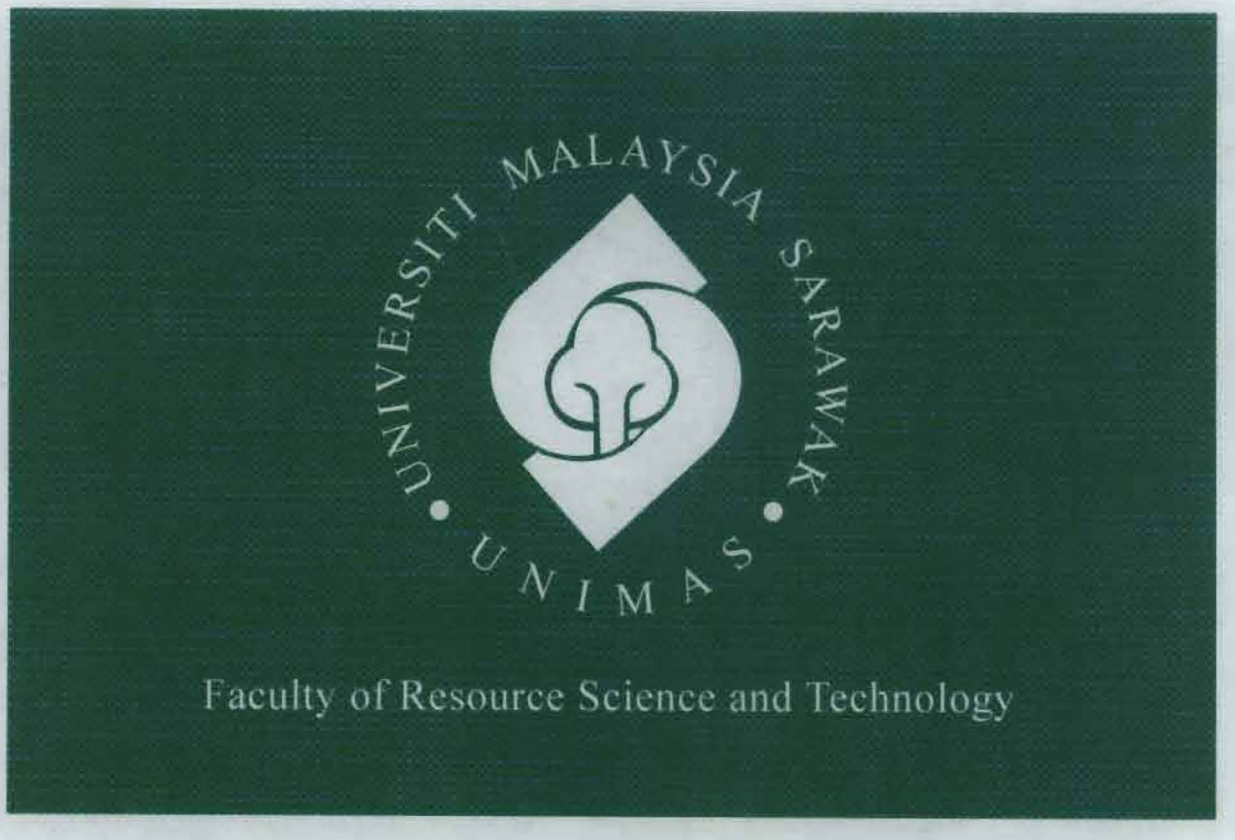

ISOLATION AND IDENTIFICATION OF LISTERIA *MONOCYTOGENES* FROM RAW VEGETABLES IN KUCHING AND KOTA SAMARAHAN, SARAWAK

Ardianshah Bin Amirudin

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ISOLATION AND IDENTIFICATION OF *LISTERIA MONOCYTOGENES* FROM RAW VEGETABLES IN KUCHING AND KOTA SAMARAHAN, SARAWAK

ARDIANSHAH BIN AMIRUDIN
(20680)

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours
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DECLARATION

I declare that the study titled Isolation and identification of *Listeria monocytogenes* from raw vegetables in Kuching and Kota Samarahan, Sarawak is my original work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. It has been submitted and shall not be submitted in any form to any institution or other university.

Ardianshah Bin Amirudin Date

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

BLEB	Buffer *Listeria* Enrichment Broth
g	gram
HCl	Hydrochloric acid
H_2S	Hydrogen Sulfide
L. grayi	*Listeria grayi*
L. innocua	*Listeria innocua*
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
L. seeligeri	*Listeria seeligeri*
L. welshimeri	*Listeria welshimeri*
ml	millilitre
MR	Methyl Red
NaCl	Sodium chloride
spp.	species
TSA	Tryptic Soy Agar
VP	Voges-Proskauer
%	percent
°C	degree Celcius
µl	micro liter
µm	micrometer
α	alpha
β	beta

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Isolation and identification of *Listeria monocytogenes* from raw vegetables in Kuching and Kota Samarahan, Sarawak

Ardianshah bin Amirudin

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

L. monocytogenes is an opportunistic intracellular pathogen that has become an important cause of human foodborne infections worldwide. In this study, *L. monocytogenes* was isolated and identified from raw vegetable samples. Four main vegetables (cucumber, cabbage, winged bean and carrot) studied were purchased from supermarkets and wet markets in Kuching and Kota Samarahan area. The technique involved in the methodology used were enrichment with BLEB, followed by isolation on PALCAM agar and CHROMagar *Listeria*, culturing on TSA and finally identification by Gram staining and conventional biochemical tests. Based on isolation of *L. monocytogenes* on PALCAM agar, the occurrence percentage of the bacteria differed slightly between the samples from supermarkets and wet markets in Kuching and Kota Samarahan which are 32.5% and 33.75%, respectively. However, following isolation on CHROMagar *Listeria*, only six positive results (7.5%) were obtained from both supermarket and wet market. Conventional biochemical tests were performed to further identify the presumptive colonies. MR-VP test, motility test and Gram staining gave positive results but indole test and H_2S production test shown negativity.

Keywords: *L. monocytogenes*, PALCAM agar, CHROMagar *Listeria* and conventional biochemical tests

ABSTRAK

L. monocytogenes merupakan patogen intrasel yang bersifat oportunis yang telah menjadi penyebab penting dalam jangkitan bawaan makanan manusia di seluruh dunia. Dalam kajian ini, L. monocytogenes telah diasing dan dikenal pastikan dari sampel-sampel berasaskan sayur mentah. Antara sayuran yang dikaji ialah timun, kubis, kacang botol dan lobak merah. Keempat-empat sayuran ini diperoleh dari pasar raya dan pasar basah di sekitar kawasan Kuching dan Kota Samarahan, Sarawak. Teknik-teknik yang digunakan termasuk pengayaan dengan BLEB, pengasingan di atas agar PALCAM dan CHROMagar Listeria, pengkulturan di atas TSA dan akhir sekali pengesanan melalui ujian biokimia piawai. Berdasarkan pengasingan L. monocytogenes di atas agar PALCAM, peratusan penemuan bakteria di dalam sample sayuran dari pasar raya dan pasar basah berbeza sedikit iaitu 32.5% dan 33.75%. Namun, setelah pengasingan dilakukan di atas CHROMagar Listeria, hanya enam sampel sayuran sahaja (7.5%) diperolehi dari kedua-dua pasar raya dan pasar basah. Ujian-ujian biokimia piawai dilakukan untuk pengecaman koloni jangkaan yang lebih terperinci. Ujian MR-VP, ujian pergerakan dan pewarnaan Gram menunjukkan keputusan positif manakala ujian indole dan ujian penghasilan H_2S menunjukkan keputusan negatif.

Kata kunci: L. monocytogenes, agar PALCAM, CHROMagar Listeria dan ujian biokimia piawai

CHAPTER 1

INTRODUCTION

1.1 Introduction

Listeria is a genus of Gram-positive bacteria containing six species namely *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii* and *L. grayi*. Among these species, *L. monocytogenes* is known to be pathogenic to human, causing listeriosis, which is one of the most virulent food borne diseases (Awaisheh, 2009). *L. monocytogenes* is a rod shape, facultative intracellular bacterium, which is considered a zoonotic pathogen (David and Odeyemi, 2007). The bacterium is widely distributed in nature such as soil and vegetables, and food processing environments such as surfaces, machine handling and floors (Awaisheh, 2009). Recent estimate suggest that approximately 2500 human listeriosis cases occur annually in the US alone, where 500 deaths are reported (Mead *et al.*, 1999; Shaila *et al.*, 2010). *L. monocytogenes* principally causes meningitides, encephalitis, and abortion in pregnant women and septicemia in newborn (Shaila *et al.*, 2010).

Raw vegetables have been reported to be prone to contamination by *L. monocytogenes* (Szabo *et al.*, 2000; Carrasco *et al.*, 2008). Even industrial process like sanitized washing to produce ready-to-eat (RTE) vegetables does not guarantee the total elimination of this pathogen (Carrasco *et al.*, 2008). Considerable research had been carried out in relation to disinfectants and solutions which reduce a hypothetical load of *Listeria* cells (Beuchat *et al.*, 2004; Carrasco *et al.*, 2008), but no treatment has been shown

to eliminate them completely, as reported in prevalence studies of *L. monocytogenes* (Szabo *et al.*, 2000). Recently, Franz and others (2010) have published a risk assessment for *L. monocytogenes* in leafy green vegetables consumed at salad bars, focusing on the pathogen growth in the supply chain and the restaurant. Another study done by Little *et al.* (2007) also reported that *L. monocytogenes* had been detected in RTE mixed salads in the United Kingdom.

In Malaysia, the presence of *L. monocytogenes* would become public concern since several types of vegetables are consumed raw in the popular dish *ulam* (Jeyaletchumi *et al.*, 2010). Vegetables are purchased from either wet market or supermarkets locally. In local wet market, a variety of food types including seafood, poultry, meat, fresh vegetables and RTE food are usually sold. According to Chai *et al.* (2007), supermarket or hypermarket generally offer foods under conditions that appear more hygienic than those in wet markets as foodstuffs and are packed before display. However, the hygienic condition and foodstuff handling methods in the packing facilities may be poor.

1.2 Objectives

The objectives of this research are:

1) To isolate *L. monocytogenes* in raw vegetables purchased from Kuching and Kota Samarahan, Sarawak by using PALCAM agar and CHROMagar *Listeria*.
2) To identify and confirm *L. monocytogenes* isolates isolated from raw vegetables through a series of biochemical tests.

CHAPTER 2

LITERATURE REVIEW

2.1 *Listeria* spp.

Listeria is a Gram-positive, non-sporulating, catalase positive, oxidase negative rod, which measures 0.5 μm in diameter and 1 - 2 μm in length. Bacterial stains show that the cells can be found in chains or as single rods. Growth of the organism on bacteriological media is enhanced by the presence of glucose or other fermentable sugars but is also dependent on the atmosphere and temperature in which they are grown. The organism can grow over a wide range of pH (4.3-9.6), water activity (~0.83) and salt concentrations (up to 10%) (Labbe and Garcia, 2001). *Listeria* are aerobic, microaerophilic and facultatively anaerobic and can be cultured over a wide temperature range (Ryser and Marth, 2007).

In 1994, Hudson and colleagues had been reported that the organism has a growth temperature range of approximately 1 °C – 45 °C, making it both psychrotrophic and a mesophilic. There are however, growth factors which are temperature dependent. For example, at 20-25 °C peritrichous flagella are formed and cause the organism to be motile, whereas at 37 °C the organism is weakly or non-motile (Galsworthy *et al.*, 1990; Ryser and Marth, 2007). Additionally, its ability to not only survive but to grow as a psychrotroph makes this pathogen unique from other commonly found food borne pathogens which are usually inhibited from growth at refrigeration temperatures (Salihu *et al.* 2008).

The genus *Listeria* currently contains six species which are *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*. According to Ryser and Marth (2007), all *Listeria* species are phenotypically very similar but can be differentiated by using a series biochemical tests such as hemolysis, reduction of nitrates to nitrites and acid production from D-xylose, L-rhamnose, alpha methyl-D-mannoside, and mannitol. The characterization of *Listeria* species is summarized in Table 2.1.

Table 2.1 Characterization of *Listeria* species (Goldman and Green, 2008)

Characteristic	*L. monocytogenes*	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*
β-hemolysis	+	+	+	-	-
Fermentation of:	-	-	-	-	-
Mannitol	-	+	+	-	+
Xylose	+	-	-	±	±
Rhamnose					
Phatogenic in human	Yes	Extremely rare	Extremely rare	No	No

Based on recent studies, *L. monocytogenes* and *L. innocua* was reported to be closely related and have confirmed that within the 16S ribosomal ribonucleic acid, only two of 1281 base pairs differ between the two species (Wang *et al.*, 1991; Czajka *et al.*, 1993; Labbe and Garcia, 2001). *L. ivanovii* is mainly responsible for abortion in animals, along with one report of a case of human illness caused by *L. seeligeri* (Hof and Rocourt, 1992; Labbe and Garcia, 2001). Other previous studies reported that, *L. innocua* and *L. welshimeri* are not capable of causing illness. Of the six species, only *L. monocytogenes* is generally regarded as causing agent for human illness (Labbe and Garcia, 2001).

2.2 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, facultative anaerobic, non-spore-forming, motile at 20-25 °C and rod-shaped bacterium with a low G+C content (Ryser and Marth, 2007). A recent report indicated that *L. monocytogenes* is non-motile and produce little or no detectable flagellin at 37 °C (Kathariou *et al.*, 1995). According to Liu (2006), *L. monocytogenes* is an opportunistic intracellular pathogen that has become an important cause of human food borne infections worldwide. *L. monocytogenes* is tolerant to extreme condition such as low pH (4.1-9.6), low temperature (about 1 - 45 °C) and high salt conditions (Hege *et al.*, 2000; Sleator *et al.*, 2003; Liu *et al.*, 2005). In general, this microorganism is found in raw milk, soft cheeses, fresh and frozen meat, poultry, seafood, fruits, and vegetable products (Rudolf *et al.*, 2001). *L. monocytogenes* has been shown to survive in food for long periods. The classification of *L. monocytogenes* is shown in Table 2.2.

Table 2.2 Classification of *L. monocytogenes* (Goldman and Green, 2008)

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Listeriaceae
Genus	*Listeria*
Species	*L. monocytogenes*

L. monocytogenes strains can be differentiated based on serology analysis. According to Jeyaletchumi *et al.* (2010), at least 13 serotypes have been recognized in *L. monocytogenes*, which include ½a, ½b, ½c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Strains

of *Listeria* species are divided into serotypes based on the basis of specific heat-stable somatic (O) and heat-labile flagellar (H) antigens.

2.3 Listeriosis

Ingestion of food contaminated with *L. monocytogenes* can result in listeriosis, a severe infectious disease, with a high fatality rate of 30% (Cocolin *et al.*, 2002). Listeriosis predominantly affects certain risk groups including pregnant women, newborn babies, elderly people and immunocompromised patients including HIV positive patients. Most healthy individuals only experience flu-like symptoms (Rodriguez-Lazaro et *al.*, 2004).

Listeriosis, is clinically defined when the organism is isolated from blood, cerebrospinal fluid and even in placenta and fetus in abortion cases. The manifestation of listeriosis includes septicemia, meningitis or meningoencephalitis, encephalitis and intrauterine or cervical infections in pregnant women which may result in spontaneous abortions or still birth. The onset of the aforementioned disorders is usually preceded by influenza-like symptoms including persistent fever followed by nausea, vomiting and diarrhea, particularly in patients who use antacid or cimetidine (Tominaga *et al.*, 2006).

Jeyaletchumi *et al.* (2010) noted that 98% of documented human listeriosis cases are due to *L. monocytogenes* serotypes ½a, ½b and 4b, whereas serotypes 4a and 4c are rarely associated with outbreaks of the disease.

2.4 Foodborne outbreaks caused by *L. monocytogenes*

Foodborne transmission of listeriosis was first documented in 1981 during a Canadian outbreak in Nova Scotia (Montville and Matthews, 2008). These outbreak infections resulted in 7 adult and 34 perinatal cases including 5 fetal deaths and 4 stillbirths. In June of 1985, Jalisco-brand Mexican-style cheese was implicated as the vehicle of infection in an outbreak of listeriosis in Southern California. In addition, a total of 142 cases were reported that involving 93 pregnant women or their offspring and 49 non-pregnant immunocompromised adults were documented in Los Angeles. From these cases, 48 deaths were recorded, accounting for a mortality rate of 33.8% (Labbe and Garcia, 2001; Montville and Matthews, 2008).

In 2008, a large, multi-province outbreak of listeriosis associated with ready-to-eat meat products contaminated with *L. monocytogenes* serotype ½a occurred in Canada was reported by Gilmour *et al.* (2010). In 2005, 10 cases of listeriosis in a small area of Switzerland were due to locally made and distributed soft cheese (Bille *et al.*, 2006). In 2006, the Czech Republic experienced one large outbreak, involving 78 patients, of whom 13 died also caused by contamination of soft cheese by *L. monocytogenes* (Vit *et al.*, 2007). In 2008, Austria experienced an outbreak of febrile gastroenteritis, including three cases of invasive listeriosis associated with jellied pork contaminated with *L. monocytogenes* (Pichler *et al.*, 2009).

2.5 Vegetables as vehicle transmission of bacterial

According to Abadias *et al.* (2008), fresh fruits and vegetables are essential components of the human diet and there is considerable evidence of the health and nutritional benefits associated with the consumption of fresh fruit and vegetables. However, fresh vegetables can be a vehicle for the transmission of bacterial, parasitic and viral pathogens capable of causing human illness and a number of reports referred to raw vegetables harboring potential food borne pathogens (Ryser and Marth, 2007). Mukherjee *et al.* (2006) reported the incidence of food borne outbreaks in Minnesota, USA caused by contaminated fresh fruit and vegetables has increased in recent years. Table 2.3 below shows types of vegetables from which *L. monocytogenes* has been isolated.

Table 2.3 Vegetables from which *L. monocytogenes* has been isolated (Anonymous, 2003)

Country	Food
Australia	Lettuce
Brazil	Cabbage, lettuce, parsley, watercress
Costa Rica	Cabbage salad
Germany	Legumes, mushrooms, salads, vegetables foods
Italy	Celery, lettuce, fennel
Japan and Korea	Vegetables and raw vegetables
Switzerland	Salads, vegetables
United State	Broccoli, cabbage, celery, cucumbers, green beans, kelp, kidney beans, lettuce, mung beans

2.6 Isolation of *L. monocytogenes*

The first step in the isolation of *L. monocytogenes* is the use of selective enrichment of Buffered *Listeria* Enrichment Broth (BLEB). This selective enrichment is effective and

improve the detection of *L. monocytogenes* and recovery of cells from various environmental stress and injuries (Wong, 2002). PALCAM agar and CHROMagar *Listeria* was selective medium agar used to identify *L. monocytogenes* (Reissbrodt, 2004).

PALCAM (polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol) agar was formulated by Netten *et al.* in 1989 to overcome the insufficient selectivity of media used in the past for the detection and enumeration of *Listeria.* For example, the studies based on the work of Rodriguez in 1984, reported the use of esculin and iron salts to visualize *Listeria monocytogenes* by its esculinase-positive character (Reissbordt, 2004). Many selective media for *Listeria* containing esculin, however, also enable the growth of several groups of *Streptococci* and they concluded that the use of esculin was limited. Among the previous selective media used, PALCAM agar give satisfactory results, producing highly typical *Listeria* colonies while at the same time inhibiting almost all other contaminating bacteria (Lee *et al.*, 2011).

CHROMagar *Listeria* is another selective medium for the isolation, differentiation and identification of presumptive *L. monocytogenes* from food and environmental samples. U.S Food and Drug Administration (2003) recommended this medium for the detection and enumeration of *L. monocytogenes* in food. Suspected *L. monocytogenes* growth on this agar is blue colonies with white halo.

In 2011, by Lee *et al.* reported CHROMagar *Listeria* and PALCAM agar play essential roles in the detection of *Listeria* spp. and *L. monocytogenes*. Hegde *et al.* (2007) further stated that CHROMagar *Listeria* had a sensitivity of 99% and 100% for the detection of *L. monocytogenes* from 200 natural and artificially inoculated food samples,

respectively, with a colony confirmation rate of 100%. The sensitivity of non-chromogenic selective media such as PALCAM for the detection of *L. monocytogenes* from these same samples was 97–99% with colony confirmation rates of 65–67.5%. From environmental samples, the *L. monocytogenes* confirmation rate was 100% for CHROMagar *Listeria* as compared to 50% for conventional agars tested. The authors also concluded that CHROMagar *Listeria* offered a high degree of specificity for the confirmation of suspected *L. monocytogenes* colonies, whereas PALCAM agar were not differential for *L. monocytogenes* from other *Listeria* species.

2.7 Gram staining and biochemical tests for the confirmation of *L. monocytogenes*

L. monocytogenes is a gram positive bacterium, thus, this organism appear as blue or purple rod-shaped cells after Gram staining when viewed under the microscope (Labbe and Garcia, 2001). Upon isolation, *L. monocytogenes* can be identified through a series of biochemical tests such as motility, Methyl red, Voges-Proskauer, H_2S production and indole test were also used to identify *L. monocytogenes* isolates (Spicer, 2000). Table 2.4 below shows the result for typical biochemical tests on *L. monocytogenes*.

Table 2.4 Biochemical characteristics of the *L. monocytogenes* (Spicer, 2000)

Characteristics	Reaction
Motility	+
Methyl red reaction	+
Voges-Proskauer reaction	+
H_2S production	-
Indole test	-

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

A total of 80 raw vegetables such as carrot, cabbage, cucumber, and winged bean were purchased from local supermarkets and wet markets in Kuching and Kota Samarahan, Sarawak (Refer to Table 3.1). The samples were purchased from October of 2010 until January of 2011 with the total sampling of seven. The samples were transported to the laboratory for analysis.

Table 3.1 Vegetable samples examined in the study

English name	Scientific name	Number of samples examined
Cabbage	*Brasssica oleracea*	20
Carrot	*Daucus carota*	20
Cucumber	*Cucumis sativus*	20
Winged bean	*Psophocarpus tetragonolobus*	20
Total		80

3.2 Enrichment

25 g of each samples were weighed under sterile condition, followed by homogenization using stomacher in 225 ml of Buffer *Listeria* Enrichment Broth (BLEB). The samples were then incubated for 4 hours at 30 °C. Ten-fold dilutions of each samples were prepared from 10^{-2} to 10^{-4} dilution.

3.3 Isolation of *L. monocytogenes*

100 µl of each dilution were directly plated onto PALCAM agar and were incubated for 48 hours at 30 °C. Then, at least 5 presumptive colonies (black with grey zone) were picked and streaked onto CHROMagar *Listeria* using sterile inoculating loop. The plates were then incubated at 37 °C for 18-24 hours.

3.4 Identification of *L. monocytogenes*

Three to five blue colonies with an opaque, white halo were picked and transferred to Tryptic Soy Agar (TSA) and incubated overnight at 37 °C. Gram staining and a series of biochemical tests were carried out to identify the isolated bacteria. The biochemical tests listed below were performed as according to Spicer, (2000).

3.4.1 Gram staining

The glass slides were initially washed with sterile distilled water and flamed using Bunsen burner before used. Once completely dried, a drop of sterile distilled water was added to each slide. A single colony from Tryptic Soy Agar (TSA) culture was inoculated and mixed evenly with sterile distilled water by gentle spreading. The slides were then flamed to retain the colonies on the surface.

Next, the slides were brought to the sink for staining by using Gram reagents. Firstly, three drops of crystal violet was added to the colonies for about one minute then